Supporting Information

for

Fluorescent Derivatives of Phenylalanine Suitable for Protein Modification

Shengxi Chen, Nour Eddine Fahmi, Chandrabali Bhattacharya, Lin Wang, Yuguang Jin, Stephen J. Benkovic and Sidney M. Hecht

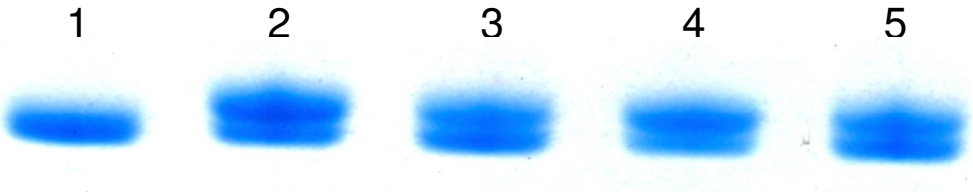
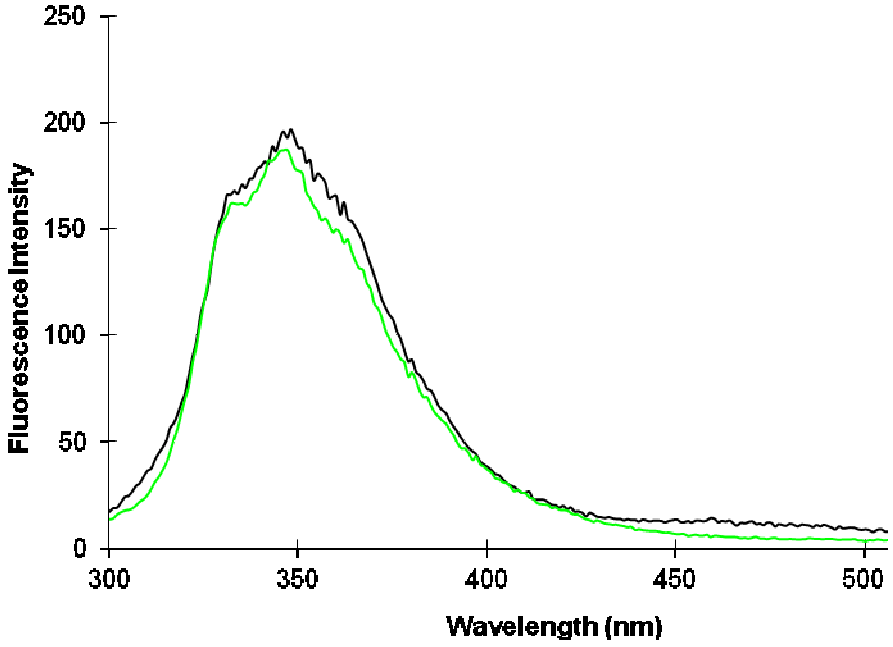


Figure S1. T4 RNA ligase-mediated ligation of $tRNA_{CUA}$-C_{OH} (lane 1) with the *N*-pentenoyl-protected pdCpA esters of amino acids **A** (lane 2), **B** (lane 3), **C** (lane 4) and **D** (lane 5).

Figure S2. Purification of DHFR containing amino acid **A** at position 16 by successive chromatographies on Ni-NTA and DEAE-Sepharose CL-6B columns. Lane 1, flow through from Ni-NTA column; lane 2, elution with 10 mM imidazole; lane 3, first elution with 150 mM imidazole; lane 4, second elution with 150 mM imidazole; lane 5, third elution with 150 mM imidazole; lane 6, flow through from the DEAE-Sepharose CL-6B column; lane 7, eltuion with 100 mM NaCl; lane 8, elution with 200 mM NaCl; lane 9, first elution with 300 mM NaCl; lane 10, second elution with 300 mM NaCl; lane 11, third elution with 300 mM NaCl.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Fluorescence Intensity
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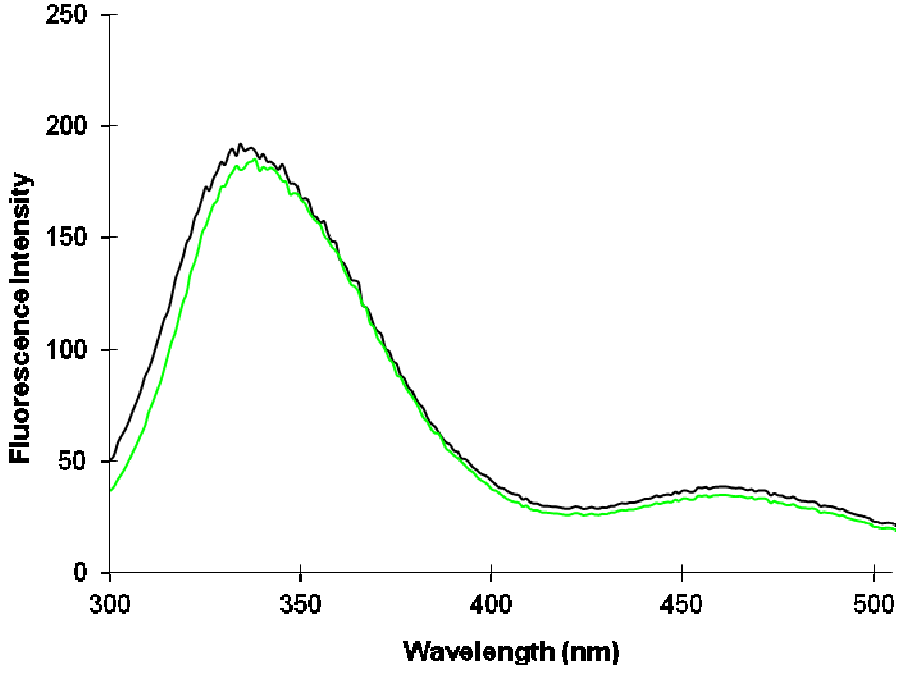
Wavelength (nm)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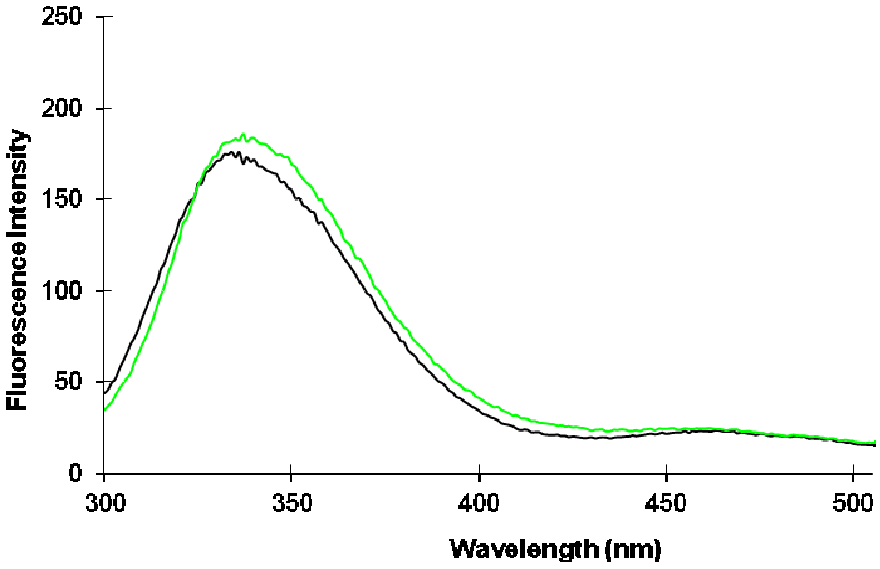
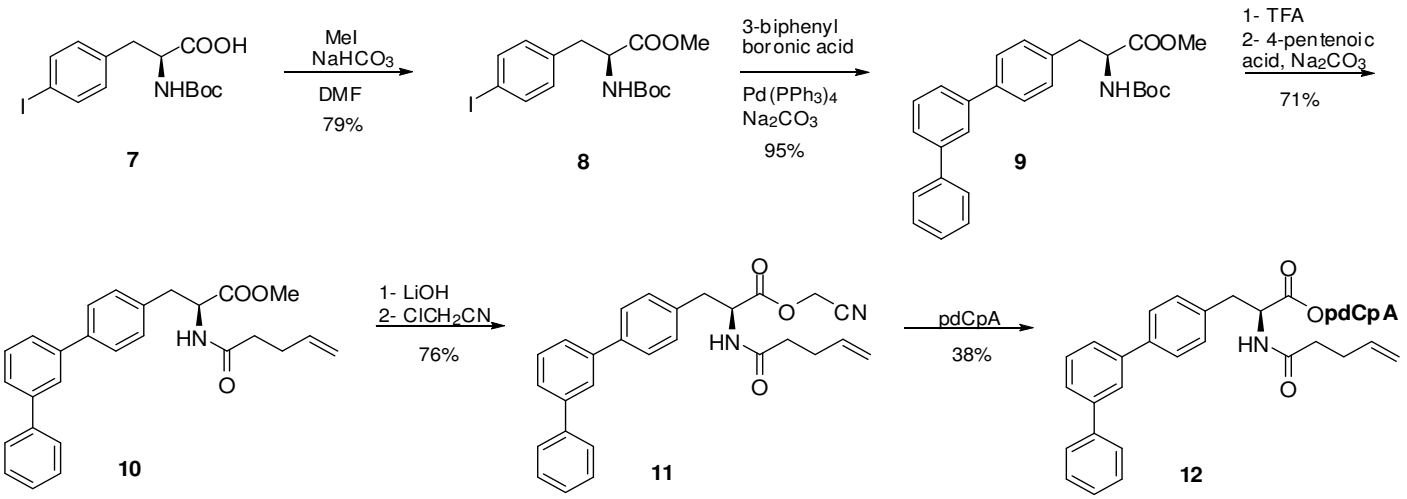
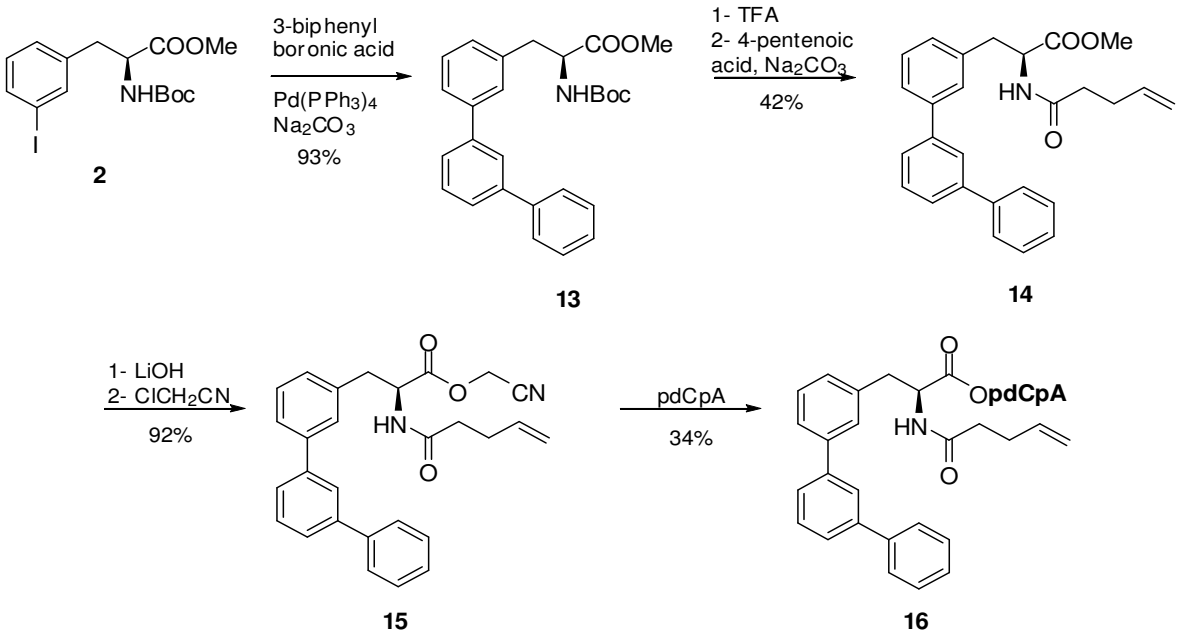


Figure S3. Fluorescence emission spectra of DHFR containing amino acids at positions 16 (black trace) and 49 (green trace). Top panel, amino acid **A**; middle panel, amino acid **C**; lower panel, amino acid **D**.



Scheme S1. Synthetic route employed for the preparation of biphenyl-phenylalanyl-pdCpA **12**.



Scheme S2. Synthetic route employed for the preparation of biphenyl-phenylalanyl-pdCpA **16**.

Experimental Section

General Methods

All non-aqueous reactions were carried out in oven-dried glassware under a balloon-pressure of argon or nitrogen. The reagents were commercially available and were used as received; anhydrous solvents were purchased as the highest grade available from Sigma-Aldrich or purified as following: THF was distilled from Na-benzophenone, CH_2Cl_2 was distilled from CaH_2. Reactions were monitored by thin layer chromatography using 0.25 mm Silicycle or EM silica gel 60 F_{254} plates. Flash column chromatography was performed using Silicycle 40-60 mesh silica gel. Yields were reported as isolated yields of spectroscopically pure compounds. ^1H and ^{13}C NMR spectra were obtained using a 400 MHz Varian spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^1H resonance of the solvent ($CDCl_3$, 7.26 ppm; DMSO-d_6, 2.49 ppm, CD_3OD, 3.31 ppm or acetone-d_6, 2.05 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.16 ppm; DMSO-d_6, 39.52 ppm, CD_3OD, 49.00 ppm or acetone-d_6, 29.84 ppm). Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were acquired at the Arizona State University High Resolution Mass Spectrometry Facility or Michigan State University Mass Spectrometry Facility.

Ni-NTA agarose was obtained from Qiagen Inc. DNA oligonucleotides were purchased from Integrated DNA Technologies. DEAE-Sepharose, ammonium persulfate, acrylamide, *N*, *N´*-methylene-bis-acrylamide, acetic acid, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phospho(enol)pyruvate, *Escherichia coli* tRNA, isopropyl β-D-

thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin, and formamide were obtained from Sigma-Aldrich. Tris and SDS were obtained from Bio-Rad Laboratories (Hercules, CA). [^{35}S]-methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from PerkinElmer Inc. Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. T4 RNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs Inc.

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lamdba 20 UV/vis spectrometer. Fluorescence was monitored using a Varian Cary Eclipse Fluorescence Spectrophotometer.

_N_-(_tert_-Butoxycarbonyl)-3-iodo-L-phenylalanine Methyl Ester (2)

To a solution containing 1.00 g (2.55 mmol) of _N_-(_tert_-butoxycarbonyl)-3-iodo-L-phenylalanine (**1**) in 12 mL of dry DMF was added 282 mg (3.36 mmol) of $NaHCO_3$ followed by 0.60 mL (1.36 g, 9.60 mmol) of iodomethane. The reaction mixture was stirred at room temperature under nitrogen for 47 h. EtOAc (30 mL) was added and the organic layer washed with three 20-mL portions of water. The organic layer was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (14 x 4 cm), eluting with 7:3 hexanes–acetone. _N_-(_tert_-Butoxycarbonyl)-3-iodo-L-phenylalanine methyl ester (**2**) was obtained as a yellow syrup which solidified upon standing: yield 792 mg (76%); silica gel TLC R_f 0.34 (7:3 hexanes–acetone); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.94 (dd, 1H, J = 13.8 and 6.2 Hz), 3.06 (dd, 1H, J = 13.8 and 5.8 Hz), 3.70 (s, 3H), 4.53 (m, 1H, J = 7.6 and 6.0 Hz), 4.98 (d, 1H, J = 7.6 Hz), 7.00 (t, 1H, J = 7.8 Hz), 7.08 (d, 1H, J = 7.6 Hz), 7.46 (s, 1H) and

7.55 (d, 1H, J = 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ28.4, 38.0, 52.5, 54.4, 80.2, 94.5, 128.6, 130.3, 136.2, 138.5, 138.7, 155.1 and 172.1; mass spectrum (APCI), *m/z* 406.0523 $(M+H)^+$ ($C_{15}H_{21}NO_4I$ requires 406.0516).

_N_-(*tert*-Butoxycarbonyl)-[3-(1,1',4',1")-terphenyl]-L-alanine Methyl Ester (3)

To 12 mL of 1:1 THF–toluene containing 100 mg (0.24 mmol) of *N*-(*tert*-butoxycarbonyl)-3-iodo-L-phenylalanine methyl ester (**2**) and 101 mg (0.51 mmol) of 4-biphenylboronic acid was added a solution of 54.0 mg (0.51 mmol) of Na_2CO_3 in 5 mL of water. The mixture was degassed by bubbling N_2 through for 30 min, then 14.5 mg (12.5 μmol) of $Pd(PPh_3)_4$ was added. The reaction mixture was stirred vigorously at 80 ºC for 20 h. Silica gel TLC (4:1 hexanes–ethyl acetate, developed twice) showed that the reaction was complete. The cooled reaction mixture was diluted with 25 mL of water and extracted with three 20-mL portions of EtOAc. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (19 × 4 cm), eluting with 9:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-[3-(1,1',4',1")-terphenyl]-L-alanine methyl ester (**3**) was obtained as a colorless thick syrup which solidified upon standing: yield 101 mg (95%); silica gel TLC R_f 0.22 (4:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ1.42 (s, 9H), 3.13 (dd, 1H, J = 13.8 and 6.2 Hz), 3.21 (dd, 1H, J = 13.8 and 5.4 Hz), 3.74 (s, 3H), 4.65 (q, 1H, J = 14.0 and 6.0 Hz), 5.03 (d, 1H, J = 8.4 Hz), 7.12 (d, 1H, J = 7.6 Hz), 7.38 (m, 3H), 7.46 (m, 2H), 7.53 (m, 1H) and 7.64–7.69 (m, 6H); mass spectrum (APCI), *m/z* 432.2178 $(M+H)^+$ ($C_{27}H_{30}NO_4$ requires 432.2175).

_N_-(4-Pentenoyl)-[3-(1,1',4',1")-terphenyl]-L-alanine Methyl Ester (4)

To a solution containing 261 mg (0.60 mmol) of *N*-(*tert*-Butoxycarbonyl)-[3-(1,1',4',1")-terphenyl]-L-alanine methyl ester (**3**) in 15 mL of dichloromethane was added 3 mL of TFA. The reaction mixture was stirred at room temperature for 4 h. The solvent was concentrated under diminished pressure and the residual TFA was removed by coevaporation with 15 mL of toluene. The crude product was obtained as an off-white solid and was used directly in the next step. The crude residue was suspended in a solution containing 127 mg (1.20 mmol) of Na_2CO_3 in 6 mL of water. 4-Pentenoic acid succinimidyl ester (130 mg, 0.66 mmol) in 6 mL of DMF was added and the reaction mixture was stirred at room temperature for 20 h. The mixture was filtered through a Celite pad, washing with two 10-mL portions of EtOAc. The filtrate was washed successively with 15 mL of 1 N aq $NaHSO_4$ and 15 mL of water. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (16 × 2.5 cm). Step gradient elution with 10 → 60% EtOAc in hexanes as eluant gave *N*-(4-pentenoyl)-[3-(1,1',4',1")-terphenyl]-L-alanine methyl ester (**4**) as an off-white solid: yield 108 mg (43%); mp 86–88 °C; silica gel TLC R_f 0.30 (7:3 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.29 (m, 2H), 2.35 (m, 2H), 3.17 (dd, 1H, J = 13.8 and 5.8 Hz), 3.24 (dd, 1H, J = 14.0 and 6.0 Hz), 3.74 (s, 3H), 4.93–5.03 (m, 3H), 5.75 (m, 1H), 6.02 (d, 1H, J = 7.6 Hz), 7.09 (d, 1H, J = 7.6 Hz), 7.37 (m, 3H), 7.46 (m, 2H), 7.53 (m, 1H) and 7.62–7.69 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.5, 35.7, 38.1, 52.5, 53.1, 115.8, 126.0, 127.1, 127.5, 127.6, 128.1, 128.3, 128.9, 129.2, 136.5, 136.9, 139.8, 140.4, 140.7, 142.0, 172.0 and 172.3; mass spectrum (APCI), *m/z* 414.2082 $(M+H)^+$ ($C_{27}H_{28}NO_3$ requires 414.2069).

_N_-(4-Pentenoyl)-[3-(1,1',4',1")-terphenyl]-L-alanine Cyanomethyl Ester (5)

To a solution containing 79.0 mg (0.19 mmol) of *N*-(4-pentenoyl)-[3-(1,1',4',1")-terphenyl]-L-alanine methyl ester (**4**) in 4 mL of THF was added a solution containing 23.0 mg (0.57 mmol) of $LiOH{\cdot}1H_2O$ in 2.5 mL of water. The reaction mixture was stirred at room temperature for 2 h. Silica gel TLC analysis, development with 7:3 hexanes–EtOAc, indicated that the reaction was complete. The mixture was acidified to pH ~3 with 1 N $NaHSO_4$ (~1.2 mL) and then extracted with 10 mL of EtOAc. The organic layer was washed with 5 mL of water, dried ($MgSO_4$) and concentrated under diminished pressure. The crude acid was dissolved in 6 mL of dry acetonitrile and 133 μL (96 mg, 0.95 mmol) of triethylamine was added followed by 121 μL (144 mg, 1.91 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 15 h. Silica gel TLC analysis of the reaction mixture (1:1 hexanes–EtOAc or 9:1:0.01 CH_2Cl_2–MeOH–AcOH) showed that the reaction was not complete. Additional triethylamine (133 μL, 0.95 mmol) and chloroacetonitrile (60 μL, 0.85 mmol) were added and stirring was continued for another 4 h. The reaction mixture was diluted with 15 mL of dichloromethane, then washed successively with 15 mL of 1 N $NaHSO_4$ solution and 15 mL of water. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (15 × 2.5 cm), eluting with 1:1 hexanes–EtOAc. *N*-(4-Pentenoyl)-[3-(1,1',4',1")-terphenyl]-L-alanine cyanomethyl ester (**5**) was obtained as a colorless solid: yield 70 mg (83%); mp 140–142 °C; silica gel TLC R_f 0.43 (1:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.30 (m, 4H), 3.18 (dd, 1H, J = 14.0 and 6.8 Hz), 3.25 (dd, 1H, J = 14.0 and 6.2 Hz), 4.69, 4.80 (ABq, 2H, J = 15.8 Hz), 4.98 (m, 3H), 5.74 (m, 1H), 5.98 (d, 1H, J = 7.6 Hz), 7.13 (d, 1H, J = 7.6 Hz), 7.37–7.48 (m, 5H), 7.57 (d, 1H, J = 8.0 Hz) and 7.64–7.70 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.3, 35.4, 37.7, 49.1, 53.0, 113.9, 116.0,

126.4, 127.1, 127.6, 127.7, 127.9, 128.1, 129.0, 129.5, 135.7, 136.7, 139.5, 140.5, 140.6, 141.4, 170.6 and 172.3; mass spectrum (APCI), *m/z* 439.2025 $(M+H)^+$ ($C_{28}H_{27}N_2O_3$ requires 439.2022).

_N_-(4-Pentenoyl)-[3-(1,1',4',1'')-terphenyl]-L-alanine pdCpA Ester (6)

To a conical vial containing a 16.0 mg (36.5 μmol) of *N*-(4-pentenoyl)-[3-(1,1',4',1'')-terphenyl]-L-alanine cyanomethyl ester (**5**) in 50 μL of anhydrous DMF was added a solution of 7.00 mg (5.14 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 40 μL of anhydrous DMF followed by 10 μL of triethylamine. The reaction mixture was stirred at room temperature for 18 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 65% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 1.2 mL with 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column. *N*-(4-Pentenoyl)-[3-(1,1',4',1'')-terphenyl]-L-alanine pdCpA ester (**6**) (retention time 31.5 min) was recovered from the appropriate fractions as a colorless solid by lyophilization: yield 1.8 mg (34%); mass spectrum (ESI), *m/z* 1018.2874 $(M+H)^+$ ($C_{45}H_{50}N_9O_{15}P_2$ requires 1018.2902).

_N_-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine Methyl Ester (8)[1,2]

To a solution containing 1.00 g (2.55 mmol) of *N*-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine (**7**) in 12 mL of dry DMF was added 282 mg (3.36 mmol) of $NaHCO_3$ followed by 0.60 mL (1.36 g, 9.60 mmol) of iodomethane. The reaction mixture was stirred at room temperature under nitrogen for 40 h. EtOAc (30 mL) was added and the organic layer washed with three 20-mL

portions of water. The organic layer was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (12 × 4 cm), eluting with 1:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (**8**) was obtained as a colorless solid: yield 0.82 g (79%); mp 78−80 °C (lit.[1] mp 78−80 °C and lit.[2] 78.5−80 °C); silica gel TLC R_f 0.66 (1:1 hexanes–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.97 (dd, 1H, J = 13.6 and 6.0 Hz), 3.07 (dd, 1H, J = 13.8 and 5.8 Hz), 3.71 (s, 3H), 4.56 (m, 1H), 4.96 (d, 1H, J = 7.2 Hz), 6.87 (d, 2H, J = 8.0 Hz) and 7.61 (d, 2H, J = 8.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4, 38.0, 52.4, 54.3, 80.2, 92.6, 131.4, 135.9, 137.7, 155.1 and 172.2; mass spectrum (MALDI), *m/z* 427.86 $(M+Na)^+$ (theoretical *m/z* 428.03).

_N_-(_tert_-Butoxycarbonyl)-[4-(1,1',3',1")-terphenyl]-L-alanine Methyl Ester (9)

To 24 mL of 1:1 THF–toluene containing 200 mg (0.48 mmol) of *N*-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (**8**) and 202 mg (1.02 mmol) of 3-biphenylboronic acid was added a solution of 108 mg (1.02 mmol) of Na_2CO_3 in 10 mL of water. The mixture was degassed by bubbling N_2 through for 30 min, then 29.0 mg (25.0 μmol) of $Pd(PPh_3)_4$ was added. The reaction mixture was stirred vigorously at room temperature for 13 h, after which silica gel TLC (4:1 hexane–ethyl acetate, developed twice) showed the reaction to be nearly complete. The reaction mixture was heated to 80 ºC and stirred for another for 3 h. The cooled reaction mixture was diluted with 40 mL of water and extracted with three 25-mL portions of EtOAc. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (23 × 4 cm), eluting with 9:1 → 4:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-[4-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**9**) was obtained as an orange syrup: yield 202 mg (95%); silica gel TLC R_f 0.23 (4:1 hexanes–EtOAc); ^{1}H NMR (400 MHz,

$CDCl_3$) δ1.42 (s, 9H), 3.10 (dd, 1H, *J* = 13.6 and 6.0 Hz), 3.18 (dd, 1H, *J* = 13.8 and 5.8 Hz), 3.74 (s, 3H), 4.63 (q, 1H, *J* = 13.6 and 5.6 Hz), 5.02 (d, 1H, *J* = 8.0 Hz), 7.22 (d, 2H, *J* = 7.6 Hz), 7.37 (m, 1H), 7.44–7.52 (m, 3H), 7.54–7.59 (m, 4H), 7.64 (m, 2H) and 7.78 (br s, 1H); mass spectrum (APCI), *m/z* 432.2173 $(M+H)^+$ ($C_{27}H_{30}NO_4$ requires 432.2175).

_N_-(4-Pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine Methyl Ester (10)

To a solution containing 188 mg (0.43 mmol) of *N*-(*tert*-butoxycarbonyl)-[4-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**9**) in 10 mL of dichloromethane was added 2 mL of trifluoroacetic acid (TFA). The reaction mixture was stirred at room temperature for 2.5 h. The solvent was concentrated under diminished pressure and the residual TFA was removed by coevaporation with two 3-mL portions of toluene. The crude product was obtained as a slightly brown solid and was used directly in the next step. The crude residue was dissolved in 3 mL of DMF and a solution containing 91.0 mg (0.86 mmol) of Na_2CO_3 in 6 mL of water was added, followed by a solution of 94 mg (0.47 mmol) of 4-pentenoic acid succinimidyl ester in 3 mL of DMF. The reaction mixture was stirred at room temperature for 20 h, diluted with 25 mL of EtOAc and washed with 15 mL of water. The organic layer was washed with 15 mL of 1 N $NaHSO_4$, dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (15 × 2.5 cm). Step gradient elution with 10 → 50% EtOAc in hexanes gave *N*-(4-pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**10**) as a colorless syrup: yield 128 mg (71%); silica gel TLC R_f 0.24 (7:3 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ2.30 (m, 2H), 2.38 (m, 2H), 3.15 (dd, 1H, *J* = 14.0 and 5.6 Hz), 3.22 (dd, 1H, *J* = 14.0 and 6.0 Hz), 3.76 (s, 3H), 4.93–5.07 (m, 3H), 5.79 (m, 1H), 6.01 (d, 1H, *J* = 8.0 Hz), 7.18 (d, 2H, *J* = 8.0 Hz), 7.37 (m, 1H), 7.44–7.59 (m, 7H), 7.65 (m, 2H) and 7.72 (t, 1H, *J* = 1.6 Hz);

^{13}C NMR (100 MHz, $CDCl_3$) δ29.5, 35.7, 37.6, 52.5, 53.1, 115.8, 126.0, 126.1, 126.3, 127.4, 127.5, 127.6, 128.9, 129.3, 129.9, 135.2, 136.9, 140.1, 141.2, 141.3, 141.9, 172.0 and 172.2; mass spectrum (APCI), *m/z* 414.2063 $(M+H)^+$ ($C_{27}H_{28}NO_3$ requires 414.2069).

_N_-(4-Pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine Cyanomethyl Ester (11)

To a solution containing 100 mg (0.23 mmol) of *N*-(4-pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**10**) in 5 mL of THF was added a solution containing 29.0 mg (0.69 mmol) of $LiOH{\cdot}1H_2O$ in 3 mL of water. The reaction mixture was stirred at room temperature for 1.5 h. Silica gel TLC analysis, development with 7:3 hexanes–EtOAc, showed that the reaction was complete. The reaction mixture was acidified to pH ~3 with 1 N $NaHSO_4$ (~1.5 mL) and then extracted with 15 mL of EtOAc. The organic layer was washed with 10 mL of water, dried ($MgSO_4$) and concentrated under diminished pressure. The crude acid was dissolved in 8 mL of dry acetonitrile and 320 μL (230 mg, 2.30 mmol) of triethylamine was added, followed by 145 μL (173 mg, 2.30 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 15 h. The mixture was diluted with 15 mL of EtOAc, then washed successively with 10 mL of 1 N $NaHSO_4$ and 10 mL of water. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (16 × 2.5 cm), eluting with 1:1 hexanes–EtOAc. *N*-(4-Pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine cyanomethyl ester (**11**) was obtained as a colorless syrup which solidified upon standing: yield 77 mg (76%); silica gel TLC R_f 0.45 (1:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ2.34 (m, 4H), 3.16 (dd, 1H, J = 14.0 and 6.6 Hz), 3.22 (dd, 1H, J = 14.0 and 6.0 Hz), 4.70, 4.81 (ABq, 2H, J = 15.6 Hz), 5.01 (m, 3H), 5.78 (m, 1H), 6.01 (d, 1H, J = 7.6 Hz), 7.23 (d, 2H, J = 8.0 Hz), 7.37 (m, 1H), 7.45–7.66 (m, 9H) and 7.79 (t, 1H, J = 1.6 Hz); ^{13}C NMR

(100 MHz, $CDCl_3$) δ29.3, 35.4, 37.3, 49.0, 52.9, 113.9, 116.0, 126.0, 126.1, 126.4, 127.3, 127.6, 127.8, 128.9, 129.4, 129.7, 134.3, 136.7, 140.5, 141.0, 141.1, 170.5 and 172.3; mass spectrum (ESI), *m/z* 439.2025 $(M+H)^+$ ($C_{28}H_{27}N_2O_3$ requires 439.2022).

_N_-(4-Pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine pdCpA Ester (12)

To a conical vial containing 15 mg (34.2 μmol) of *N*-(4-pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine cyanomethyl ester (**11**) in 50 μL of anhydrous DMF was added a solution of 7 mg (5.14 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 40 μL of anhydrous DMF followed by 10 μL of triethylamine. The reaction mixture was stirred at room temperature for 15 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 65% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 0.9 mL with 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column. *N*-(4-Pentenoyl)-[4-(1,1',3',1")-terphenyl]-L-alanine pdCpA ester (**12**) (retention time 30.8 min) was recovered from the appropriate fractions as a colorless solid by lyophilization: yield 2 mg (38%); mass spectrum (ESI), *m/z* 1018.2924 $(M+H)^+$ ($C_{45}H_{50}N_9O_{15}P_2$ requires 1018.2902).

_N_-(*tert*-Butoxycarbonyl)-[3-(1,1',3',1")-terphenyl]-L-alanine Methyl Ester (13)

To 24 mL of 1:1 THF–toluene containing 210 mg (0.51 mmol) of *N*-(*tert*-butoxycarbonyl)-3-iodo-L-phenylalanine methyl ester (**2**) and 205 mg (1.03 mmol) of 3-biphenylboronic acid was added a solution of 110 mg (1.03 mmol) of Na_2CO_3 in 10 mL of water. The mixture was

degassed by bubbling N_2 through for 30 min, then 30.0 mg (25.0 μmol) of $Pd(PPh_3)_4$ was added and the reaction mixture was stirred vigorously at 80−85 ºC under a nitrogen atmosphere. The cooled reaction mixture was diluted with 40 mL of water and extracted with three 25-mL portions of EtOAc. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (14 × 4 cm), eluting with 4:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-[3-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**13**) was obtained as an orange syrup: yield 209 mg (93%); silica gel TLC R_f 0.29 (4:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 9H), 3.13 (dd, 1H, J = 13.8 and 6.2 Hz), 3.22 (dd, 1H, J = 14.0 and 5.6 Hz), 3.73 (s, 3H), 4.65 (q, 1H, J = 14.0 and 6.4 Hz), 5.04 (d, 1H, J = 8.0 Hz), 7.14 (d, 1H, J = 7.2 Hz), 7.37−7.59 (m, 9H), 7.65 (m, 2H) and 7.77 (t, 1H, J = 1.6 Hz); mass spectrum (APCI), *m/z* 432.2182 $(M+H)^+$ ($C_{27}H_{30}NO_4$ requires 432.2175).

_N_-(4-Pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine Methyl Ester (14)

To a solution containing 180 mg (0.41 mmol) of *N*-(*tert*-butoxycarbonyl)-[3-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**13**) in 10 mL of dichloromethane was added 2 mL of TFA. The reaction mixture was stirred at room temperature for 3 h. The solvent was concentrated under diminished pressure and the residual TFA was removed by coevaporation with 5 mL of toluene. The crude product was obtained as an off-white solid and was used directly in the next step. The crude residue was dissolved in 3 mL of DMF. A solution containing 91.0 mg (0.86 mmol) of Na_2CO_3 in 6 mL of water was added followed, by a solution containing 94.0 mg (0.47 mmol) of 4-pentenoic acid succinimidyl ester in 3 mL of DMF. The reaction mixture was stirred at room temperature for 15 h, diluted with 25 mL of EtOAc and washed with 15 mL of water. The organic layer was washed with 15 mL of a 1 N $NaHSO_4$ solution, dried ($MgSO_4$) and

concentrated under diminished pressure. The crude residue was purified on a silica gel column (16 ×2.5 cm). Step gradient elution with 10 → 50% EtOAc in hexanes afforded *N*-(4-pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**14**) as a colorless syrup: yield 73 mg (42%); silica gel TLC R_f 0.26 (7:3 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.31 (m, 4H), 3.16 (dd, 1H, *J* = 14.0 and 6.0 Hz), 3.25 (dd, 1H, *J* = 14.0 and 5.8 Hz), 3.73 (s, 3H), 4.96 (m, 3H), 5.73 (m, 1H), 6.12 (d, 1H, *J* = 7.6 Hz), 7.11 (d, 1H, *J* = 7.6 Hz), 7.38 (m, 3H), 7.45–7.60 (m, 6H), 7.65 (m, 2H) and 7.77 (t, 1H, *J* = 1.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.4, 35.6, 38.0, 52.4, 53.1, 115.7, 126.09, 126.12, 126.3, 127.3, 127.5, 128.31, 128.33, 128.9, 129.1, 129.3, 136.5, 136.8, 141.1, 141.4, 141.5, 141.9, 172.0 and 172.2; mass spectrum (APCI), *m/z* 414.2075 $(M+H)^+$ ($C_{27}H_{28}NO_3$ requires 414.2069).

_N_-(4-Pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine Cyanomethyl Ester (15)

To a solution containing 66.0 mg (0.15 mmol) of *N*-(4-pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine methyl ester (**14**) in 4 mL of THF was added a solution containing 19 mg (0.45 mmol) of $LiOH{\cdot}1H_2O$ in 2 mL of water. The reaction mixture was stirred at room temperature for 2 h. Silica gel TLC analysis, development with 1:1 hexanes–EtOAc, showed that the reaction was complete. The reaction mixture was acidified to pH ~3 with 1 N $NaHSO_4$ (~1 mL) and extracted with 10 mL of EtOAc. The organic layer was washed successively with 5 mL of water, 5 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The crude acid was dissolved in 4 mL of dry acetonitrile and 212 µL of triethylamine (152 mg, 1.50 mmol) was added followed by 95.0 µL (114 mg, 1.5 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen at room temperature for 17 h, diluted with 10 mL of EtOAc, and then washed successively with 7 mL of 1 N aq $NaHSO_4$ and 7 mL of water. The organic layer was dried

($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (14 × 2.5 cm), eluting with 1:1 hexanes–EtOAc. *N*-(4-Pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine cyanomethyl ester (**15**) was obtained as a colorless solid: yield 62 mg (92%); mp 110−112 °C; silica gel TLC R_f 0.35 (1:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.30 (m, 4H), 3.17 (dd, 1H, J = 14.0 and 6.8 Hz), 3.26 (dd, 1H, J = 14.2 and 6.2 Hz), 4.68, 4.78 (ABq, 2H, J = 15.6 Hz), 4.96 (m, 3H), 5.72 (m, 1H), 5.99 (d, 1H, J = 7.6 Hz), 7.14 (d, 1H, J = 7.6 Hz), 7.36−7.61 (m, 9H), 7.65 (m, 2H) and 7.77 (br t, 1H, J = 1.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.4, 35.4, 37.7, 49.0, 53.0, 113.9, 116.0, 126.2, 126.5, 126.6, 127.3, 127.6, 128.1, 128.2, 128.9, 129.4, 129.5, 135.8, 136.6, 141.1, 141.2, 141.9, 142.9, 170.5 and 172.3; mass spectrum (APCI), *m/z* 439.2018 $(M+H)^+$ ($C_{28}H_{27}N_2O_3$ requires 439.2022).

_N_-(4-Pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine pdCpA Ester (16)

To a conical vial containing 16.0 mg (36.5 μmol) of *N*-(4-pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine cyanomethyl ester (**15**) in 50 μL of anhydrous DMF was added a solution of 7.00 mg (5.14 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 50 μL of anhydrous DMF followed by 10 μL of triethylamine. The reaction mixture was stirred at room temperature for 20 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 65% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 1.2 mL with 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column. *N*-(4-Pentenoyl)-[3-(1,1',3',1")-terphenyl]-L-alanine pdCpA ester (**16**) (retention time 30.6 min) was recovered from the appropriate fractions as a

colorlesssolid by lyophilization: yield 1.8 mg (34%); mass spectrum (ESI), *m/z* 1018.2922 $(M+H)^+$ ($C_{45}H_{50}N_9O_{15}P_2$ requires 1018.2902).

Ligation of Suppressor $tRNA_{CUA}$-C_{OH} with L-(7-Hydroxycoumarin-4-yl)ethylglycinyl-pdCpA

L-(7-hydroxycoumarin-4-yl)ethylglycinyl-pdCpA was prepared as reported previously.[3,4] Ligation of suppressor $tRNA_{CUA}$-C_{OH} with L-(7-hydroxycoumarin-4-yl)ethylglycinyl-pdCpA was carried out in 300 μL (total volume) of 100 mM Hepes buffer, pH 7.5, containing 2.0 mM ATP, 15 mM $MgCl_2$, 300 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of N-protected aminoacyl-pdCpA (5-10 fold molar excess), 15% DMSO and 600 units of T4 RNA ligase. After incubation at 37 ^oC for 1 h, the reaction was quenched by the addition of 30 μL of 3 M NaOAc, pH 5.2, followed by 900 μL of ethanol. The reaction mixture was incubated at –20 ^oC for 30 min, then centrifuged at 15,000 × g at 4 ^oC for 30 min. The supernatant was carefully decanted and the tRNA pellet was washed with 300 μL of 70% ethanol, and dissolved in 60 μL of RNase free H_2O.

Ligation of Suppressor $tRNA_{CCCG}$-C_{OH} with Biphenyl-phenylalanyl-pdCpA

The suppressor $tRNA_{CCCG}$-C_{OH} was prepared as previously reported.[5-7] Ligation of suppressor $tRNA_{CCCG}$-C_{OH} was carried out in 100 μL (total volume) of 100 mM Hepes buffer, pH 7.5, containing 2.0 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of N-pentenoyl protected biphenyl-phenylalanyl-pdCpA (5-10 fold molar excess), 15% DMSO and 200 units of T4 RNA ligase. After incubation at 37 ^oC for 1 h, the reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixture

was incubated at –20 °C for 30 min, then centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was carefully decanted and the tRNA pellet was washed with 100 μL of 70% ethanol, and dissolved in 80 μL of RNase free H_2O. The N-pentenoyl protecting group was removed by treatment with 5 mM I_2 at room temperature for 10 min. The reaction mixture was treated with 10 μL of 0.3 M NaOAc, pH 5.2. followed by 300 μL of ethanol. After centrifugation at 15,000 × g at 4 °C for 30 min, the supernatant was decanted carefully and the tRNA pellet was washed with 100 μL of 70% ethanol, and then dissolved in 35 μL of RNase free H_2O.

In vitro Translation of DHFR Analogues Using a Plasmid Containing a TAG Codon at Position 115

The modified DHFR plasmid containing a TAG codon at position 115 was obtained by site-directed mutation as described previously using the wild-type DHFR plasmid as the template.[8] The DNA primer for the mutation at position 115 was 5'-CAAAAACTGTATCTGACGCATTAGGACGCAGAAGTGGAAGGCGAC-3'.

The *in vitro* expression mixture (300 μL total volume) contained 30 μg of mutant DHFR (TAG at position 115) plasmid DNA, 120 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM maganesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP and 4 mM cAMP), 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 90 μg of biphenyl-phenylalanyl-$tRNA_{CCCG}$ and 90 μL of S-30 extract from *E. coli* strain BL21(DE3). The reaction mixture was incubated at 37 °C for 45 min. Plasmid DNA containing the gene for wild-type DHFR was used as the positive control, and an abbreviated tRNA ($tRNA\text{-}C_{OH}$) lacking any amino acid was used as the negative

control. An aliquot containing 2 μL of reaction mixture was removed, treated with 2 μL of loading buffer and heated at 90 ^{o}C for 2 min. This was analyzed by 15% SDS-PAGE, run at 100 V for 2 h.

***In vitro* Translation of DHFR Analogues Using a Plasmid Containing a CGGG Codon at Position 17 and a TAG Codon at Position 115**

The modified DHFR plasmid containing a four-base CGGG codon at position 17 and a TAG codon at position 115 was obtained by site-directed mutation as described as previously using the wild-type DHFR plasmid as the template.[8] The DNA primer for the mutation at position 17 was 5'-GTAGATCGCGTTATCGGCATGCGGGAACGCCATGCCGTGGAACCTG-3'; the primer for the mutation at position 115 was 5'-CAAAAACTGTATCTGACGCATTAGGACGCAGAAGTGGAAGGCGAC-3'.

The *in vitro* expression mixture (300 μL total volume) contained 30 μg of modified DHFR plasmid (CGGG at position 17 and TAG at position 115) plasmid DNA, 120 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM maganesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP and 4 mM cAMP), 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 150 μg of L-(7-hydroxycoumarin-4-yl)ethylglycinyl-tRNA$_{CUA}$, 90 μg of biphenyl-phenylalanyl-tRNA$_{CCCG}$ and 90 μL of S-30 extract from *E. coli* strain BL21(DE3). The reaction mixture was incubated at 37 ^{o}C for 45 min. Plasmid DNA containing the gene for wild-type DHFR was used as the positive control, and an abbreviated tRNA (tRNA-C$_{OH}$) lacking any amino acid was used as the negative control. An aliquot containing 2 μL of reaction mixture was removed,

treated with 2 μL of loading buffer and heated at 90 ^{o}C for 2 min. This was analyzed by 15% SDS-PAGE at 100 V for 2 h.

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